Reference: *Biol. Bull.* **181:** 427–441. (December, 1991)

Proteins of Crustacean Exoskeletons: I. Similarities and Differences among Proteins of the Four Exoskeletal Layers of Four Brachyurans

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Abstract. Most of the proteins extracted from exocuticle, endocuticle, and membranous layer of four species of anecdysial (intermolt) crabs (the Bermuda land crab *Gecarcinus lateralis,* the rock crab *Cancer antennarius,* the shield-backed kelp crab *Pugettia producta,* and the southern shield-backed kelp crab *Taliepus nuttalli*) were 31 kDa or smaller; proteins of similar M_r were common to all three layers. Proteins from the membranous layer were qualitatively indistinguishable in all four species. More proteins 31 kDa or smaller were similar in size and pI to proteins from other exoskeletal layers than were proteins larger than 31 kDa. Proteins extracted from the epicuticle of *G. lateralis* included a group of five ranging from 54 to 42 kDa that bound $^{45}Ca^{++}$ *in vitro.* The group was not seen in other layers of the exoskeleton of *G. lateralis* or, with the exception of 44 and 42 kDa protein bands that were in the epicuticle of *C. antennarius,* in any layers of the exoskeletons of the other three species. During proecdysis, the membranous layer is completely degraded, and proteins 31 kDa or smaller are preferentially degraded from the exocuticle and endocuticle of the old exoskeleton of *G. lateralis,* which is cast as an exuvia at ecdysis. The relative amounts of proteins in extracts of epicuticle from (1) anecdysial exoskeletons and (2) exuviae were very similar, suggesting that there was little degradation of epicuticle during proecdysis. Some of the proteins of the three inner layers of the exoskeleton of *G. lateralis* have characteristics similar to those of flexible cuticles of insects; they have acidic pIs and they form "charge trains," *i.e.,* proteins of the same size separated by differences in charge during isoelectric focusing.

Received

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Introduction

Arthropods grow by molting, a programmed process of developmental changes that occurs prior to ecdysis (proecdysis) and in the period immediately following ecdysis (metecdysis). After the events of metecdysis are completed, the molting process is considered to be finished and the animal enters anecdysis, the intermolt period during which there is no further increase in external dimensions. During each proecdysial period, the epidermis underlying the exoskeleton synthesizes a new epicuticle and exocuticle, the two outermost layers of the new exoskeleton. Concomitantly, the epidermis degrades about 75% of the old exoskeleton, the remainder of which is shed as an exuvia at ecdysis (reviewed in Skinner, 1985; Stevenson, 1985; Skinner *et al.,* 1991).

The exoskeleton of an anecdysial (intermolt) decapod crustacean consists of four layers. From the outside in they are epicuticle, exocuticle, endocuticle, and membranous layer. The epicuticle is thin [about 7 μm; thicknesses are given for the Bermuda land crab *Gecarcinus lateralis* (Skinner, 1962)] and composed of proteins, lipids and calcium salts, but no chitin (Travis, 1955; Welinder, 1975b; Stevenson, 1985). The epicuticle is thought to render the exoskeleton impermeable (Mary and Krishnan, 1974), preventing both water loss and solubilization of exoskeletal components in aqueous habitats (Dennell, 1960). The exocuticle (about 30 μm) is a calcified matrix of chitin and protein. The epicuticle and exocuticle form the soft shell of the newly exuviated crab. These layers are thought to harden during metecdysis by tanning (Vacca and Fingerman, 1975a,b) and calcification (Travis,

1960). The endocuticle (about 200–400 μm) is also a calcified matrix of chitin and protein. Its synthesis and calcification begin in metecdysis, several days after ecdysis (Stage B of Drach, 1939; see Skinner, 1962), and continue through Stage C_3. The membranous layer (about 20–30 μm) also contains chitin and protein but is not calcified; it is the innermost layer and abuts the epidermis at the apical cell membrane. The membranous layer is synthesized during Stage C_3 (Green and Neff, 1972), and its completion signals the onset of anecdysis, or intermolt (stage C_4; Skinner, 1962, 1985). A discrete membranous layer has not been described in reviews of insect cuticle (Neville, 1975; Filshie, 1982; Willis, 1987).

Compared to the exoskeletons or cuticles of many other arthropods, the greater size and thickness of the decapod crustacean exoskeleton present distinct advantages for isolating individual layers of the exoskeleton and of exuviae. These characteristics of the crab exoskeleton also permit the recovery of significant amounts of protein from individual layers. It is much more difficult to separate the individual layers of the cuticles of most insects (Hackman, 1974).

Despite the recoverability of the separate layers of crustacean exoskeletons, amino acid compositions have been determined only for entire exoskeletons of the crayfish *Astacus fluviatilis* (Welinder, 1974, 1975a; Herzog *et al.*, 1975); the crabs *Scylla serrata* (Hackman, 1974), *Cancer pagurus,* and *Carcinus maenus;* the Norway lobster *Nephrops norvegicus;* and the shrimp *Penaeus duorarum* (Welinder, 1974). Tyrosine, which is a rather rare component of most proteins, constitutes 3 to 7.5% of the total amino acids in most analyses. Such a result might be expected if tyrosine is involved in protein cross-linking in crustacean exoskeletons (Vacca and Fingerman, 1975a,b). In general, exoskeletal proteins are rich in glycine, which may play a role in covalent binding of protein to chitin (Herzog *et al.*, 1975). Acidic amino acids account for approximately 20% of the total.

Intact proteins of some exoskeletons have also been analyzed. Exoskeletons have either been extracted directly and their proteins displayed on polyacrylamide gels (*S. serrata;* Hackman, 1974), or prior to analyses, fractionated into three parts: epicuticle plus exocuticle, endocuticle, and membranous layer (*C. pagurus;* Welinder, 1975b). In both of these investigations, the positions of the extracted proteins in polyacrylamide gels after electrophoresis were diagrammed, but the actual gels were not shown. The amino acid compositions of mixtures of the extracted proteins have also been determined (Hackman, 1974; Welinder, 1975b). Exoskeletal proteins of the crayfish *Astacus leptodactylus* have been sized on one dimensional polyacrylamide gels (Vranckx and Durliat, 1980, 1986). With the exception of the latter analysis, in which some of the proteins were as large as 400 kDa (Vranckx and Durliat, 1986), most of the exoskeletal proteins from Crustacea had M_r smaller than 30 kDa.

Finally, different concentrations of 20-hydroxyecdysone, selected to mimic hemolymph concentrations throughout the intermolt cycle, have been tested on the synthesis of crustacean integumentary proteins, both exoskeletal and cellular. The effects have been monitored *in vitro* in *A. leptodactylus* (Bielefeld *et al.*, 1986; Traub *et al.*, 1987) and both *in vitro* (Paulson and Skinner, 1991) and *in vivo* (Stringfellow and Skinner, 1988) in *G. lateralis.* The most significant effects were on the synthesis of small M_r proteins.

We have been investigating some of the cellular and molecular events in the molting process. They occur during proecdysis, and include synthesis of muscle in regenerating limbs concomitant with atrophy of muscle in chelae (Skinner, 1966; Mykles and Skinner, 1982, 1985), and synthesis of the two outer layers of a new exoskeleton (epicuticle and exocuticle) concomitant with the partial degradation of the old exoskeleton. The mechanisms that control such events must be complex. We focus here on the composition, synthesis, and turnover of the exoskeleton.

In this report, we describe proteins present in the four layers of brachyuran exoskeletons, with an emphasis on *G. lateralis,* the exoskeleton of which has been analyzed during several different stages of the intermolt cycle. We show electrophoretic patterns of proteins and compare the proteins present in anecdysial, proecdysial, and metecdysial exoskeletons and exuviae of *G. lateralis.* The distribution of a number of the proteins in one or more layers of the exoskeleton (data shown here), as well as the patterns of their synthesis (Stringfellow and Skinner, 1988; Paulson and Skinner, 1991) and degradation (O'Brien and Skinner, 1987, 1988), make them favorable candidates for the investigation of genes specifically activated during the molting process.

The exuvia is much more friable than the anecdysial exoskeleton. The cuticular proteins that are degraded during proecdysis may be involved in the calcification and maintenance of rigidity, whereas those that are "protected" from degradation may be more important in determining the framework and shape of the exoskeleton.

Materials and Methods

Experimental animals and preparation of layers of their exoskeletons

G. lateralis specimens obtained from the Bermuda Biological Station were maintained at approximately 25°C and 12:12 h light:dark cycle. Precocious molts were induced in *G. lateralis* by autotomy of more than four limbs (Skinner and Graham, 1970, 1972). Specimens of the rock crab *Cancer antennarius,* the shield-backed kelp crab

Pugettia producta, and the southern shield-backed kelp crab *Taliepus nuttalli,* were collected near Santa Barbara, California.

Anecdysial animals were placed on ice for 0.5–2 h to separate integumentary tissues from the membranous layer (O'Brien *et al.,* 1986). Cell debris was removed from the membranous layer, and the external surface of the carapace was cleaned with moist Kimwipes. Epicuticles from anecdysial animals were removed from the dorsal carapace with a power rotor (Li'l Crafty, Sears) equipped with a steel pyramidal bit. Both exoskeleton and rotor were held inside a plastic bag, which collected airborne particles of epicuticle as they were scraped from the carapace. The procedure was repeated to collect exocuticle. In *G. lateralis,* epicuticle (dark purple in the dorsal carapace), exocuticle (orange), and endocuticle (white), can be distinguished easily on the basis of color. Following removal of the epicuticle and exocuticle, the endocuticle was cracked and the membranous layer (ML) peeled away from the inner surface of the endocuticle; this provided ML C (membranous layer adjacent to epidermal cells; Fig. 2, lane 5). The inner layer of the endocuticle was also scraped to remove any remnants of membranous layer; this provided ML E (adjacent to endocuticle; Fig. 2, lane 4).

The possibility that particular proteins are associated with pigmented regions of the exoskeleton was determined as follows. From the same animals, proteins were extracted from the pooled three outer layers (epicuticle plus exocuticle plus endocuticle) of the highly pigmented dorsal carapaces of anecdysial crabs, and from the unpigmented regions of the branchiostegites. The branchiostegites are lateral extensions of the thoracic exoskeleton, and cover the gill chambers; their underlying integumentary tissues comprise an inner and an outer sheet of epidermis separated by a layer of connective tissue containing storage cells, tegumental glands, and hemolymph sinuses (Skinner, 1962). Proteins were also extracted from the two newly synthesized outer layers (epicuticle plus exocuticle) of unpigmented regions of branchiostegites, moderately pigmented lateral branchiostegites, and the purple dorsal region of the carapaces of metecdysial animals within a few hours following ecdysis during Stage A (Drach, 1939; Skinner, 1962). Exuviae, remnants of the old exoskeleton from which the animal emerges at ecdysis, are lined by a transparent ecdysial membrane (Stevenson, 1985) which was removed. Epicuticles, exocuticles, and endocuticles were recovered from exuviae by careful chipping with a sharp scalpel. During vacuum desiccation, pieces of epicuticle separated spontaneously and cleanly from exuviae.

Extraction of proteins

Exoskeletal layers were dried at room temperature. The three outer calcified layers were pulverized separately in a mortar and pestle, and membranous layers were minced. Samples were extracted in 5 *M* guanidine thiocyanate, 0.4 *M* EDTA, 5 m*M* Pipes, pH 7, (1:10, w:v), with gentle shaking at room temperature for 2–5 h. Extracts were centrifuged at 16,000 × *g* for 5 min in an Eppendorf microfuge, and the supernatants were recovered. Samples to be stored or electrophoresed were dialyzed against Buffer A (10 m*M* ammonium bicarbonate, pH 7.8) overnight at 4°C. Samples to be iodinated were dialyzed overnight at 4°C against 0.4 *M* NaCl, 2.5 m*M* NaN_3, 5 m*M* Pipes, pH 7. Following iodination by the chloramine T method (McConahey and Dixon, 1980), the samples were dialyzed against Buffer A at 4°C overnight until radioactivity in the dialysate was reduced to background. Protein was measured by fluorescence (Avruch and Wallach, 1971); aliquots were then lyophilized and stored at −60°C. Lyophilized proteins were solubilized in appropriate buffers for 1 or 2D gel electrophoresis. Samples for ash-free dry weights were dried to constant weight at 55°C and then heated at 500°C for 4 to 24 h in pre-ashed aluminum boats.

Gel electrophoresis

A discontinuous gel system was used for 1D gels (Laemmli, 1970). Separating gels were gradients of 5–15% polyacrylamide (Stringfellow and Skinner, 1988) or 9–18% polyacrylamide, the latter contained 0–10% glycerol (O'Brien and Skinner, 1988); stacking gels were 4.5% acrylamide. In 2D analyses, proteins were separated in the first dimension by isoelectric focusing (IEF) using tube gels (0.8 mm × 11 cm) composed of 9 *M* urea, 3.8% acrylamide, 0.2% bis, and 5% ampholines (O'Farrell *et al.,* 1977). The ampholines were a 1:1:1 blend of pH = 3–5, 4–6, and 3–10 (Bio-Rad) that produced an approximate gradient of pH 5–7. Proteins were separated in the second dimension by size on SDS slab gels (O'Farrell, 1975). Gels were stained with silver (Wray *et al.,* 1981) or Coomassie blue. To compare the efficacy of the two stains, some gels were stained first with Coomassie blue, destained, and restained with silver. The relative intensities of bands in stained 1D gels were quantified with an LKB 2202 ULTROSCAN laser densitometer attached to an Apple II computer (scans not shown). Autoradiography was as described (Stringfellow and Skinner, 1988; Paulson and Skinner, 1991).

Detection of calcium-binding proteins

Calcium-binding by proteins in gels was detected according to the method of Maruyama *et al.* (1984). Proteins (75–100 μg/lane) extracted from specific layers of *G. lateralis* exoskeleton were separated by SDS-PAGE. Gels were equilibrated in transfer buffer (25 m*M* Tris, pH 8.5, 192 m*M* glycine, 20% methanol) with 3 (100 ml) × 20

min washes. Proteins were transferred to 0.1 μm (pore size) nitrocellulose membranes (Schleicher and Schuell) with a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) at 4°C for 16 h at 60 V. Membranes were washed with 10 m*M* imidazole-HCl, pH 6.8, 60 m*M* KCl, 5 m*M* $MgCl_2$ (Buffer B), 3 (100 ml) × 20 min then soaked 10 min in 50 ml Buffer B with 1 mCi/L $^{45}Ca^{++}$ (New England Nuclear), washed for 5 min in 500 ml 50% ethanol and dried at room temperature.

Results and Discussion

The intermolt cycle of a decapod crustacean is divided into an intermolt period (anecdysis), a premolt period (proecdysis), ecdysis, and a postmolt period (metecdysis). Two layers of the new exoskeleton, the epicuticle (outermost layer) and the exocuticle (the next inner layer) are synthesized during proecdysis. The animal emerges at ecdysis clad in these two layers only; the partially degraded old exoskeleton is shed as an exuvia. In metecdysis, synthesis of an endocuticle begins. In *G. lateralis*, formation of the endocuticle continues for a month or so after which synthesis of the innermost layer, the membranous layer, begins. The completion of the membranous layer signals the beginning of anecdysis.

Characteristics of the layers of the anecdysial (intermolt) exoskeleton

Some characteristics of the four layers of the anecdysial exoskeleton of *G. lateralis* are listed in Table I. Epicuticle constituted about 15%, exocuticle 8%, endocuticle 75%, and membranous layer only about 2% of the dry weight. The large contribution of the epicuticle may be related to its role in inhibiting water loss. Desiccation is a major influence affecting adaptation of crabs to a terrestrial environment (Wolcott, 1988) and *G. lateralis* has one of the lowest rates of evaporative water loss among brachyurans (Greenaway, 1988). Because epicuticle is only one quarter the thickness of exocuticle but has twice the dry weight, the water content of the native epicuticle must also be very low.

The concentration of extractable protein per unit mass of dry weight was lowest in the epicuticle and increased progressively with each deeper layer. Although the innermost membranous layer contributed the least to the relative weight of the exoskeleton, perhaps due to the lack of calcium salts, it yielded the most extractable protein per unit mass. Ash-free dry weights of the exoskeleton measure the relative composition of inorganic material, chiefly calcium salts in the form of calcite (Roer and Dillaman, 1984). The epicuticle and exocuticle of *G. lateralis* had slightly higher levels of inorganic material than the underlying endocuticle; the relative ash-free composition

Table I

Characteristics of layers of exoskeleton of Gecarcinus lateralis

Layer	Relative dry weight (% of total)	Protein concentration (μg protein/mg dry wt.)	Ash-free dry weight (% of pre-ash wt.)
Epicuticle	14.9	4.1	78.7
Exocuticle	8.2	8.3	76.1
Endocuticle	75.2	13.3	67.8
Membranous layer	1.7	69.3	1.9

in the three outer layers of the exoskeleton ranged from 68 to 79% of their dry weights. These levels are only slightly less than levels of calcium salts found in the four layers combined of the carapaces of the marine crabs *C. pagurus* (85.0%) and *C. maenas* (84.1%; Welinder, 1974), and are very similar to the calcium content measured for the four layers combined of the carapace of the freshwater crayfish *A. fluviatilis* (78.8%; Welinder, 1975a).

Comments on methods used for detecting proteins on gels

Each of the three common methods for visualizing proteins in polyacrylamide gels, (1) staining with Coomassie blue (Fristrom *et al.*, 1978; Cox and Willis, 1985; Cox, 1987; Kimbrell *et al.*, 1988) or (2) silver staining (Wray *et al.*, 1981; Schleicher and Watterson, 1983; O'Brien and Skinner, 1987; Stringfellow and Skinner, 1988), or (3) autoradiography of iodinated proteins (O'Brien and Skinner, 1987; 1988), has some disadvantages. Differences in staining are observed when proteins extracted from the four layers of the exoskeleton of *G. lateralis* are electrophoresed in a single gel and then stained first with Coomassie, then, after destaining, restained with silver (Fig. 1). Two to three times as many protein bands stain intensely with silver (lanes 3, 5, 7, and 9) as with Coomassie blue (lanes 2, 4, 6, and 8). Silver staining is generally a more sensitive technique (Wray *et al.*, 1981). Nevertheless, silver stains certain proteins less intensely than Coomassie. For example, a pair of bands of about 14 and 13 kDa is clearly seen in Coomassie blue-stained lanes of epicuticle, exocuticle, and endocuticle, as is a 15 kDa band in epicuticle; only trace amounts of these three protein bands are seen in the silver-stained gel (Fig. 1). Iodination of proteins is the most sensitive method. With only 0.8 to 15.2 μg iodinated proteins in 2D gels (Fig. 4A–D), a number of novel spots were seen in charge trains forming a series of spots of the same M_r spanning a range of pH.

Two series of Coomassie blue-stained 2D gels of each of the four exoskeletal layers were analyzed. In one, 20 μg protein (slightly more than the maximum amount of iodinated protein used in any of the autoradiographs

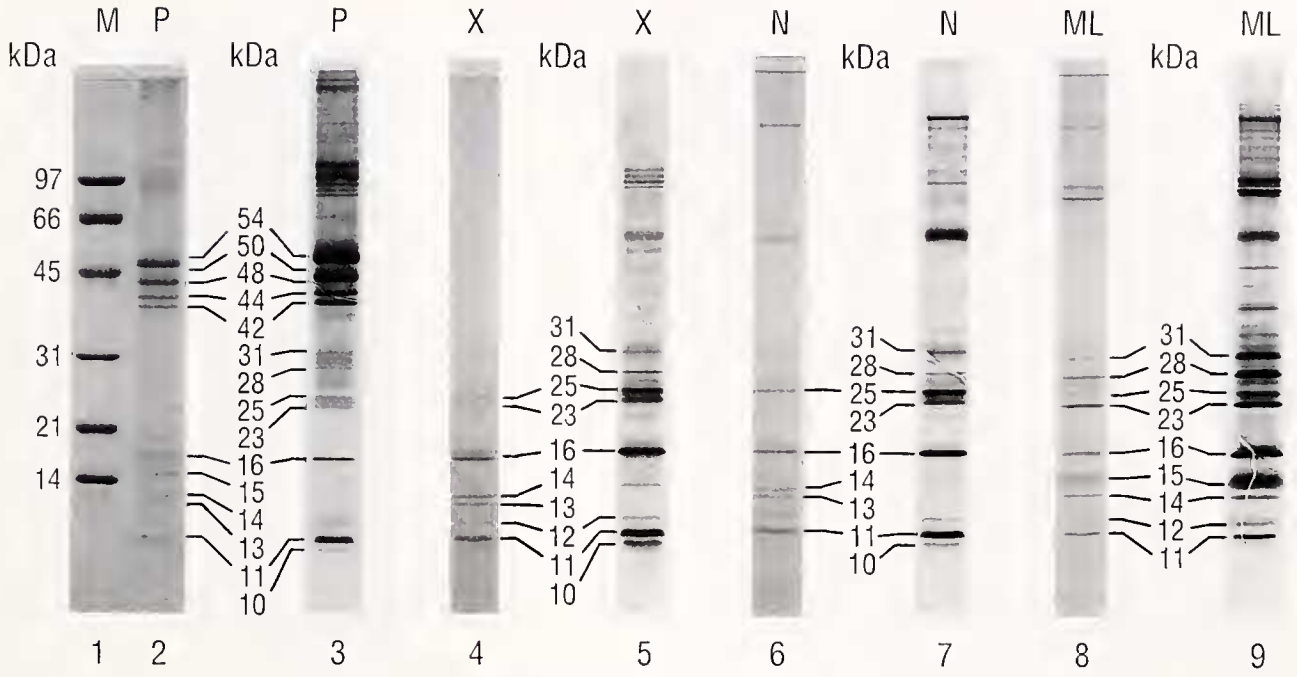

Figure 1. Comparison of *Gecarcinus lateralis* exoskeletal proteins stained with either Coomassie blue or silver. Lanes are from a 1D gel (9–18% acrylamide; 0–10% glycerol) on which was electrophoresed proteins (30 μg/lane) of each of the four exoskeletal layers of an anecdysial crab. After electrophoresis, the gel was stained with Coomassie blue, photographed, destained, then stained with silver and photographed again. The lanes from the two photographs were cut out and the two differently stained lanes of each of the four exoskeletal layers were placed adjacent to each other. Lane 1, low molecular weight standards (Bio-Rad), stained with Coomassie blue. Lanes 2–9, layers from exoskeleton; lanes 2, 3, epicuticle, P; lanes 4, 5, exocuticle, X; lanes 6, 7, endocuticle, N; lanes 8, 9, membranous layer, ML. Lanes 1, 2, 4, 6, and 8, stained with Coomassie blue; alternate lanes stained with silver. Approximate sizes of major protein bands equal or smaller than 55 kDa are indicated between the paired lanes; a dash indicates in which lane the band is present. Proteins larger than 55 kDa could not be accurately sized because a high concentration of acrylamide had been used and only low M_r standards had been run.

shown in Fig. 4) was loaded on each IEF tube gel and electrophoresed, after which the second dimension gels (9–18% acrylamide) were stained with Coomassie blue. In the gel of the epicuticle, only a group of five proteins, ranging in size from 54 to 42 kDa, referred to here as the epicuticular quintet, could be detected (data not shown). No bands were seen in the gels of the other layers even after attempts to maximally enhance visualization of the proteins.

In preparation for amino acid sequencing of individual protein spots, large amounts of protein (250 μg/gel), isolated from each of the four exoskeletal layers were electrophoresed on a second set of 2D gels. The gels were blotted on PVDF (polyvinylidene difluoride) membranes, which have a higher binding capacity than other types of membranes (Graddis, 1990). Fixation with 0.5% glutaraldehyde (Karey and Sirbasku, 1989) further increased the retention of the proteins to the filters. When such blots were stained with Coomassie blue, many spots were seen (Table III) that were not detected when smaller quantities of protein were analyzed.

Although radioiodination is the most sensitive method, the intensity of the bands observed depends on the amino acid composition of the proteins. Tyrosines, and to a lesser extent histidines, are the primary targets of iodination and proteins rich in these amino acids are the most readily visualized. In addition, iodination may cause structural modifications that may alter the electrophoretic mobilities on gels and, consequently, the banding patterns of proteins in comparison to the banding patterns of untreated proteins that are electrophoresed and then visualized by staining with either silver or Coomassie blue. Nevertheless, when populations of proteins are similarly treated, iodinated in this case, the same structural modifications should be sustained by the same proteins in each population. Thus, meaningful comparisons can be made among preparations analyzed by the same method.

Analysis of extractable proteins in anecdysial exoskeleton and exuvia by 1D gel electrophoresis

In the following, proteins of anecdysial (intermolt) animals are considered first, then proteins of proecdysial

(prior to ecdysis) and metecdysial (after ecdysis) animals. In each category, layers of the exoskeleton are described from most external to most internal, *i.e.*, epicuticle, exocuticle, endocuticle, and membranous layer.

The members of the epicuticular quintet were prominent in both anecdysial epicuticle and epicuticle of exuviae (Fig. 2, lanes 1, 6, 7). Exuviae contain epicuticle and remnants of exocuticle and endocuticle, but little if any membranous layer, most or all of which is degraded during proecdysis. The presence of the epicuticular quintet was correlated with the extent of pigmentation (see Fig. 3). Other proteins ranging from 94 to 80 kDa that were prominent in anecdysial epicuticle, exocuticle, and endocuticle were missing from epicuticle of exuviae and markedly reduced in the other two layers (Fig. 2). Other than lacking the 94–80 kDa proteins, the profiles of epicuticle protein extracts were very similar before and after ecdysis.

Extracts from exocuticle, endocuticle, and membranous layer from anecdysial *G. lateralis* contained many protein

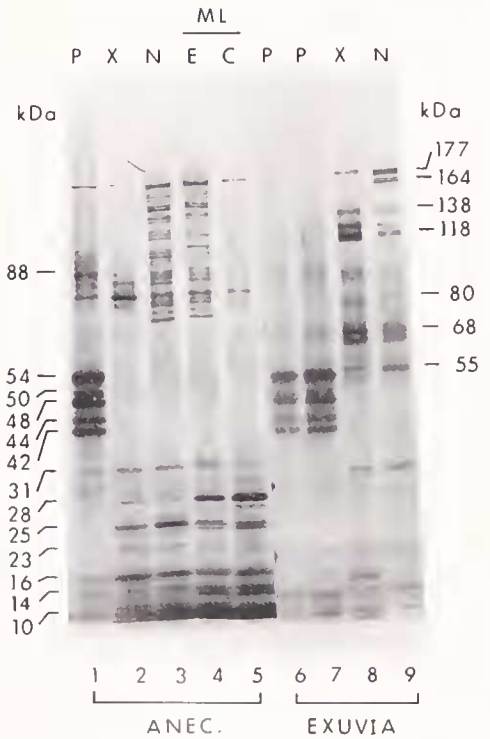

Figure 2. Proteins in anecdysial layers of the exoskeleton and layers of the exuvia of *Gecarcinus lateralis*. Silver-stained SDS gel (5–15% acrylamide), 7.5 μg protein/lane. Abbreviations as in Figure 1. Numbers to left and right of lanes indicate M_rs (kDa) of protein bands. Lanes 1–5, layers from exoskeleton of anecdysial animal. Lane 1, P; lane 2, X; lane 3, N; lane 4, membranous layer adjacent to endocuticle, ML E; lane 5, membranous layer adjacent to epidermal cells of integumentary tissues, ML C. Lanes 6–9, layers from exuvia. Lane 6, epicuticle isolated by vacuum desiccation, P; lane 7, epicuticle isolated by chipping with a scalpel, P; lane 8, X; lane 9, N.

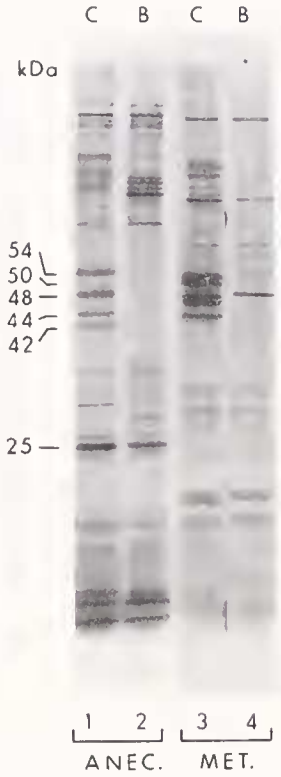

Figure 3. Epicuticular quintet occurs in pigmented regions of exoskeleton. Proteins extracted from P + X + N of the branchiostegite and dorsal carapace regions of *Gecarcinus lateralis*. SDS silver-stained gel (9–18% acrylamide, 0–10% glycerol); 7.5 μg protein/lane. C, dorsal carapace; B, branchiostegite. Numbers to left indicate M_rs (kDa) of protein bands. Lane 1, anecdysial purple dorsal carapace; lane 2, anecdysial unpigmented branchiostegite; lane 3, metecdysial purple dorsal carapace; lane 4, metecdysial unpigmented branchiostegite.

bands 31 kDa or smaller (Fig. 2, lanes 2–5). Although protein bands of these sizes formed only traces in epicuticle, many were prominent in the other three layers. While this result points up a different composition for the epicuticle, the correspondence of M_rs of proteins in the other three layers does not necessarily imply their identity.

Protein bands of 16–10 kDa were very conspicuous in both fractions of the membranous layer (ML E, closer to endocuticle, and ML C, closer to epidermal cells); low M_r bands were also conspicuous in exocuticle and endocuticle. Most of these major low M_r protein bands as well as a prominent 25 kDa band in exocuticle, endocuticle, and ML C, were present only in trace amounts or missing in extracts from exuviae (Fig. 2, lanes 6–9). As a consequence of the preferential degradation of small M_r proteins *in vivo* during proecdysis, a number of other proteins 55 kDa or larger were relatively more prominent in extracts from exocuticle and endocuticle of exuviae than from the same layers from anecdysial animals.

Protein patterns in exocuticle extracts were highly reproducible. The gel shown in Figure 1 was 9–18% acryl-

amide and 0–10% in glycerol; the gel in Figure 2 was 5–15% acrylamide. The different concentrations of acrylamide in the two gels facilitate the clear display of proteins of low M_r in Figure 1 and those of high M_r in Figure 2. Comparison of the silver-stained lanes of all four layers including the membranous layer of Figure 1 (in which ML E and ML C were not separated) with the same lanes in Figure 2 (membranous layer operationally subdivided into ML E plus ML C) indicates the presence of many bands of similar M_r. As mentioned above, many of the protein bands that stained with silver did not stain with Coomassie blue; in particular, note those larger than 16 kDa seen only as traces or not seen at all in exocuticle and endocuticle, and those larger than 28 kDa also seen only as traces or not seen at all in the membranous layer. Evidence that the protein extractions are reproducible is also seen in Figure 2, lanes 6 and 7; arrays of proteins on 1D gels of extracts of epicuticle from *G. lateralis* that had been removed from exuviae by either a scalpel or vacuum desiccation were indistinguishable from one another. Epicuticle from highly pigmented regions of anecdysial exoskeletons (Fig. 1, lanes 2 and 3; Fig. 2, lane 1; Fig. 3, lane 1), exuviae (Fig. 2, lanes 6 and 7), and metecdysial exoskeleton (Fig. 3, lane 3) of *G. lateralis*, the latter extracted one day after ecdysis, contained the proteins of the epicuticular quintet.

As previously described (O'Brien and Skinner, 1987, 1988), we have isolated from integumentary tissues two alkaline cysteine proteinase activities (ACPs) and two proteinases with acidic pH optima (APs) that preferentially degraded membranous layer proteins 26 kDa or smaller. The absence of small M_r proteins from exuviae (Fig. 2, lanes 8 and 9) suggests that some (or all) of the ACPs and APs isolated from integumentary tissues participate in the degradation of the exoskeleton of the land crab during proecdysis. Other proteinases, as yet unidentified, may also contribute to the degradation.

Correlation of the epicuticular quintet with pigmentation

The epicuticular quintet, which was not seen in extracts of any other layer, was prominent in extracts of pigmented dorsal carapace of both anecdysial (epicuticle plus exocuticle plus endocuticle combined; Fig. 3) and metecdysial animals (epicuticle plus exocuticle combined), but much less prominent in extracts of unpigmented regions of branchiostegites of anecdysial and metecdysial animals. A 48 kDa protein band conspicuous in unpigmented regions of branchiostegites from metecdysial animals (Fig. 3, lane 4) is discussed in the section on proteins from metecdysial animals, below.

Two proteins of 54 and 42 kDa are major components in extracts of integumentary tissues maintained *in vitro*, labeled with ^{3}H-tyrosine, ^{3}H-leucine, or ^{35}S-methionine and electrophoresed on polyacrylamide gels (Stringfellow and Skinner, 1988). Both proteins are labeled with both leucine and methionine at relatively high rates throughout the intermolt cycle. During the stages of proecdysis when epicuticle and exocuticle are synthesized ($D_{2,early}$ and $D_{2,late}$), incorporation of methionine increases considerably, that of leucine more than doubles, and that of tyrosine, undetectable during anecdysis and early proecdysis, is almost as high as that of the other two radiolabeled amino acids (Stringfellow and Skinner, 1988). The absence of significant stainable quantities of the 54 and 42 kDa proteins from all layers of the exoskeleton of *G. lateralis* other than epicuticle may be due to a rapid turnover of these proteins as compared to longer-lived structural proteins of the exoskeleton. Alternatively, these may not be structural proteins of the exoskeletal matrix but may be metabolically active proteins in epidermal cells of the underlying integumentary tissues or in extensions of cells within pore canals that permeate the exoskeleton (Green and Neff, 1972; Halcrow, 1976; Roer, 1980).

In addition to the quintet, a major 25 kDa protein band is seen in samples of pooled epicuticle plus exocuticle plus endocuticle from both pigmented and unpigmented regions of anecdysial crabs (Fig. 3, lanes 1 and 2). The sources of the 25 kDa protein band are, in all likelihood, exocuticle and endocuticle; note the intense staining of the 25 kDa protein in those two layers relative to that in epicuticle (Fig. 2, compare lane 1 with lanes 2 and 3). The protein is not seen immediately after ecdysis (stage A of metecdysis) even though the newly synthesized exocuticle is present. Since the protein is extractable from anecdysial exocuticle, which should be considerably more rigid than metecdysial exocuticle, it seems unlikely that the protein is not extractable because the exocuticle has begun to harden (Fig. 3, lanes 3 and 4).

Distinguishing individual proteins by 2D gel electrophoresis: anecdysial exoskeleton

Analysis of 2D gels of radioiodinated (Fig. 4; Table II) exoskeletal proteins from each layer gave a somewhat different pattern of overlaps than the analysis of 2D gels of proteins stained with Coomassie blue (Table III). Nevertheless, the principal conclusion is the same in either case: proteins 31 kDa or smaller show extensive similarity (percentage of proteins of equivalent M_r and pI) among the layers, while proteins larger than 31 kDa show very much less similarity. In any case, whether the overlapping proteins are in fact identical awaits amino acid sequencing.

Epicuticular proteins (Fig. 4A), especially those 31 kDa or smaller, displayed the most distinct pattern when compared to proteins extracted from the other three layers. Epicuticle differs from the other three layers in: (1) the presence of the epicuticular quintet (Figs. 1–3); (2) having

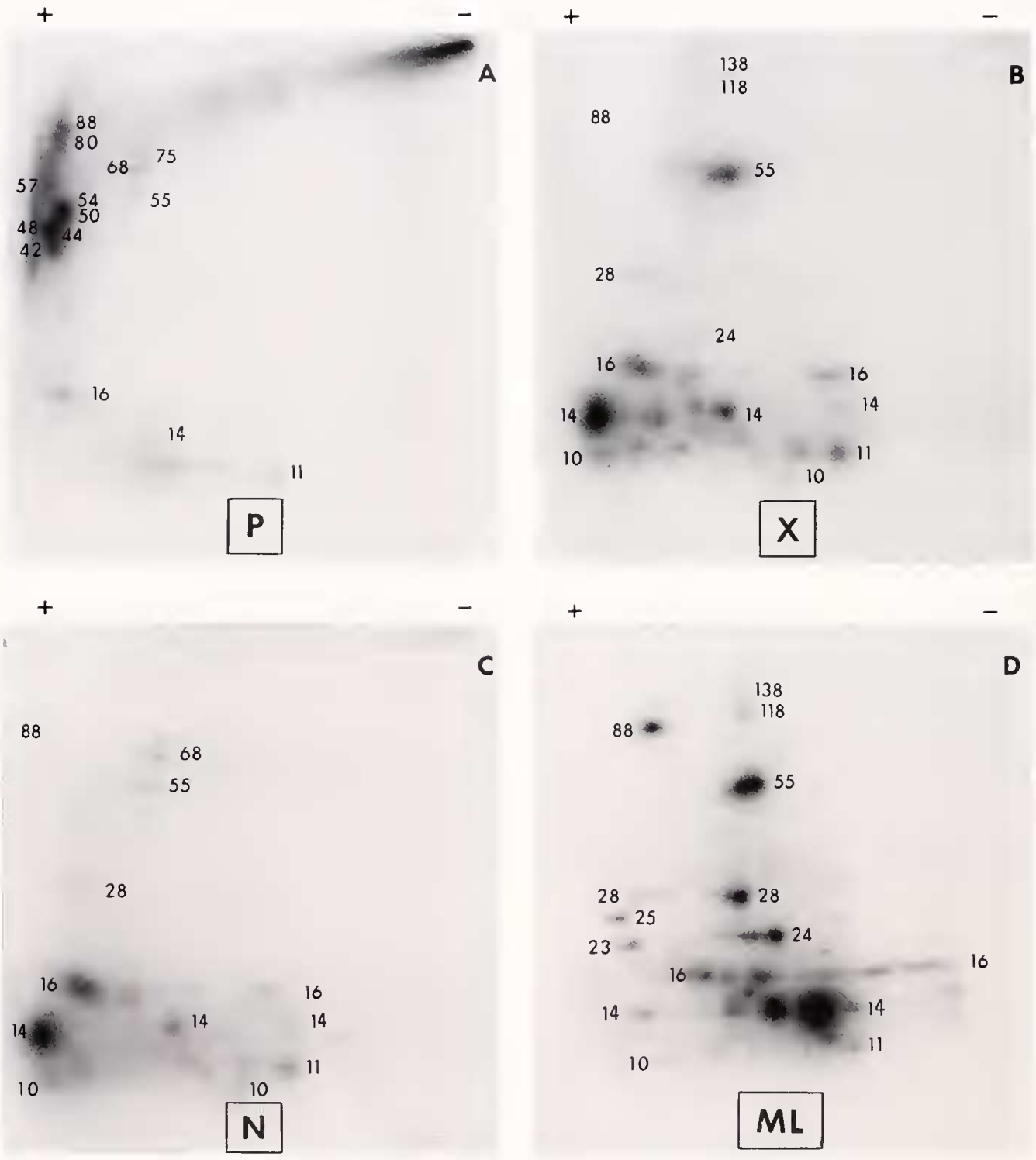

Figure 4. Analyses on 2D gels of proteins from the four layers of the anecdysial exoskeleton of *Gecarcinus lateralis*. Autoradiographs of 2D gels. SDS gel for second dimension was 9–18% polyacrylamide, 0–10% glycerol. Approximate M_rs (kDa) of proteins are indicated adjacent to spots. (A) epicuticle; 3×10^6 cpm, 0.81 μg proteins. Streak in upper right corner is an artifact. (B) As (A) except 15.2 μg exocuticle proteins. (C) As (A) except 10.6 μg endocuticle proteins. (D) As (A) except 13.2 μg membranous layer proteins. Autoradiographs A–C exposed 48 h; D exposed 42 h.

Table II

Overlap on 2D gels (similar M_r and pI) of proteins isolated from the four different layers of anecdysial Gecarcinus lateralis *exoskeleton and labeled with ^{125}I*

	All Proteins				>31 kDa				<31 kDa			
	No. spots on gel	P	X	N	ML	No. spots on gel	P	X	N	ML	No. spots on gel	P	X	N	ML
		% (no.) of overlapping spots					% (no.) of overlapping spots					% (no.) of overlapping spots			
P	27	—	52 (14)	56 (15)	44 (12)	14	—	21 (3)	28 (4)	7 (1)	13	—	85 (11)	85 (11)	85 (11)
X	49	28 (14)	—	80 (39)	71 (35)	9	33 (3)	—	56 (5)	33 (3)	40	28 (11)	—	85 (34)	80 (32)
N	44	34 (15)	89 (39)	—	84 (37)	9	44 (4)	56 (5)	—	67 (6)	35	31 (11)	97 (34)	—	88 (31)
ML	54	22 (12)	65 (35)	68 (37)	—	9	11 (1)	33 (3)	67 (6)	—	45	24 (11)	71 (32)	69 (31)	—

A transparency of an autoradiograph or blot was laid over a transparency of the autoradiograph or blot of the exoskeletal layer to which it was to be compared. The number of spots in each category (total, >31 kDa, <31 kDa) on the 2D gel from each layer is listed in the left-hand column of each of the three sections of the table. In the other boxes in the table are given the percent (number in parentheses) of spots from each exoskeletal layer that matched the spots (same M_r and pI) from the exoskeletal layer listed on the left. P, epicuticle; X, exocuticle; N, endocuticle; ML, membranous layer. Fewer spots are numbered in the 2D gels in Figure 4A–D than are listed in Table II because some spots visible in autoradiographs were lost in reproduction. In addition, intense spots in some autoradiographs were composed of more than one protein; this was verified by inspection of other autoradiographs that had shorter exposure times.

fewer proteins 31 kDa or smaller in comparison to the other layers (Tables II and III); and (3) the absence of proteins that in other layers formed charge trains on 2D gels in the pH range of the IEF gradient (compare Fig. 4A with Fig. 4B–D). The charge trains can be seen as a series of spots of the same M_r spanning a range of pH. Note that only 0.8 μg epicuticular proteins were required to obtain 3×10^6 cpm for the autoradiograph as compared to 10- to 20-times as much protein for autoradiographs of the other layers. The proteins of the epicuticular quintet may be richer in tyrosine or histidine, and therefore more heavily iodinated, than proteins from other exoskeletal layers. Nevertheless, the epicuticular quintet proteins are also major components in gels stained with silver (Fig. 1, lane 3; Fig. 2, lanes 1, 6, 7) or Coomassie blue (Fig. 1, lane 2).

Similarities and differences of proteins in the four exoskeletal layers: anecdysial

For iodinated proteins 31 kDa or smaller (Table II), the degree of similarity was 69 to 97% among extracts from the exocuticle (Fig. 4B), endocuticle (Fig. 4C), and membranous layer (Fig. 4D). Thirty-nine of the 49 proteins in exocuticle matched 39 of the 44 proteins in endocuticle. The proteins (total) in extracts of membranous layer (Fig. 4D) were also similar in size and pI to those extracted from exocuticle and endocuticle. Of 54 membranous layer proteins, 71% were similar to proteins of exocuticle and 84% to those of endocuticle.

An 88 kDa protein was prominent in extracts of membranous layer (Fig. 4D); a similar sized protein was also present in extracts of the anecdysial epicuticle, but to a lesser extent. Only traces of an 88 kDa protein were seen in the other two anecdysial layers. The 88 kDa proteins stain only lightly with silver and are thus not seen in all layers in Figures 1 and 2; they label to a very high specific activity with ^{125}I, implying a richness in tyrosine or histidine (McConahey and Dixon, 1980). They may be identical with, or related to, the 89 kDa protein that incorporated ^{35}S-methionine, ^{3}H-leucine, or ^{3}H-tyrosine as much as twice as fast during proecdysis as compared to anecdysis (Stringfellow and Skinner, 1988). Several pro-

Table III

Overlap on 2D gels (similar M_r and pI) of proteins isolated from anecdysial Gecarcinus lateralis *exoskeleton and stained with Coomassie blue*

	All Proteins				>31 kDa				<31 kDa			
	No. spots on gel	P	X	N	ML	No. spots on gel	P	X	N	ML	No. spots on gel	P	X	N	ML
		% (no.) of overlapping spots					% (no.) of overlapping spots					% (no.) of overlapping spots			
P	40	—	55 (22)	38 (15)	25 (10)	12	—	25 (3)	8 (1)	0 (0)	28	—	68 (19)	50 (14)	36 (10)
X	49	49 (24)	—	39 (19)	41 (20)	16	19 (3)	—	31 (5)	38 (6)	33	64 (21)	—	42 (14)	42 (14)
N	45	27 (12)	51 (23)	—	44 (20)	16	13 (2)	44 (7)	—	25 (4)	29	34 (10)	55 (16)	—	55 (16)
ML	45	22 (10)	44 (20)	44 (20)	—	19	0 (0)	32 (6)	21 (4)	—	26	38 (10)	54 (14)	62 (16)	—

As Table II except data are from Coomassie blue stained PVDF filters.

teins of approximately 90 kDa (PCP-28) that are specific to pupal cuticles of *M. sexta* were also labeled by ^{3}H-leucine and focused at about the same pI (Kiely and Riddiford, 1985) as did the crab protein. The 88 kDa proteins were not degraded *in vivo* or *in vitro* by proteinases endogenous to the membranous layer (see Fig. 5 in O'Brien and Skinner, 1987) and were prominent in iodinated extracts of exuviae of *G. lateralis* (data not shown).

For Coomassie blue stained proteins smaller than 31 kDa (Table III), the degree of similarity was 36 to 68% among extracts from the exocuticle, endocuticle, and membranous layer. Twenty-three of the 49 proteins in exocuticle matched 23 of the 45 proteins in endocuticle. The proteins (total) in extracts of membranous layer were also quite similar in size and pI to those extracted from exocuticle and endocuticle. Of a total of 45 membranous layer proteins, 41% were similar to proteins of exocuticle and 44% to those of endocuticle.

Charge trains in proteins of anecdysial exoskeletal layers

Proteins that are approximately the same size, but that differ in net charge, form charge trains in isoelectric focusing gels. All four layers of the exoskeleton from anecdysial crabs had proteins with acidic pIs, and some of the proteins from the three internal layers formed charge trains between pH 5 and 7 (Fig. 4). These patterns are similar to those of proteins extracted from flexible insect cuticle (Andersen *et al.*, 1986; Cox and Willis, 1987a,b). There were more charge trains in membranous layer extracts than in extracts from other exoskeletal layers; these were at 28, 25, 24, 16, 14, 11, and 10 kDa (Fig. 4D). A 28 kDa protein band, minor in both exocuticle and endocuticle (Figs. 1, 2), focused in the acidic region of the gels (Fig. 4B, C). The major component of a 28 kDa charge train in the membranous layer focused in the neutral to basic region (Fig. 4D), similar to a 27 kDa charge train in the innermost layers of *M. sexta* endocuticle (Wolfgang and Riddiford, 1986), equivalent to the crustacean membranous layer. In 1D gels stained with silver, the 25 kDa protein band was very intense; the 24 kDa band barely detectable. The 24 kDa proteins may thus be expected to be very rich in tyrosine or histidine and consequently be more highly labeled *in vitro* with ^{125}I. In fact, in integumentary tissues removed from animals at the time that epicuticle and exocuticle are being synthesized ($D_{2,early}$ and $D_{2,late}$) and incubated with ^{3}H-tyrosine, a 24 kDa protein band is very heavily labeled (Stringfellow and Skinner, 1988). The 24 kDa charge train was specific to the exocuticle and membranous layer but was present in only trace amounts in the former (Fig. 4B, D).

Proteins extracted from the inner three exoskeletal layers also had 16 and 14 kDa charge trains. The most heavily ^{125}I-labeled protein in the 14 kDa charge train in the exocuticles and endocuticles focused in the acidic region of the gel, whereas the most heavily labeled 14 kDa protein in membranous layer focused in the neutral to basic region. It appears that proteins from the membranous layer are comprised more of neutral rather than acidic amino acids. These data agree with those of Welinder (1975b), who reported fewer acidic amino acids in proteins extracted from the membranous layer than from epicuticle plus exocuticle or endocuticle. Cox and Willis (1987a) described three groups of proteins from flexible insect cuticle: (1) those with acidic pIs, (2) those that formed charge trains, and (3) those that formed elongated vertical streaks. Crab exoskeletal proteins showed similarities to the first two groups of insect proteins, but not to the third. Such streaks are observed in gels that contain urea in the resolving gel, which ours did not.

Welinder (1975b) categorized the exoskeletons of arthropods as "soft" or "hardened"; soft cuticles had a high chitin content (63–72%) and proteins that were (1) rich in acidic amino acids, (2) poor in non-polar amino acids, and (3) highly soluble. The first characteristic is one found by Willis (1987) to be true of proteins of "flexible" cuticle of insects. By contrast, hardened crustacean exoskeleton had a low chitin content and proteins that were (1) rich in nonpolar amino acids and (2) less soluble (Welinder, 1975b). Welinder observed that the exoskeletons of all crustaceans he examined were "soft," even though they are physically hard due to calcification. Our data indicate that many crustacean exoskeletal proteins are acidic, similar to those of flexible insect cuticle; thus they support Welinder's observation that crustacean exoskeleton has some of the characteristics of "soft" cuticle.

Proteins of newly synthesized exoskeleton: metecdysial

Proteins were extracted from the newly synthesized epicuticle plus exocuticle which encase the animal as it emerges at ecdysis. Those proteins that became heavily iodinated had acidic pIs; all except members of the epicuticular quintet migrated to the bottom of the IEF tube gel (Fig. 5). Although three of the ^{125}I-labeled metecdysial proteins (Fig. 5; 57, 54, and 48 kDa) had similar mobilities to proteins present in extracts of anecdysial epicuticle (Fig. 4A), none was similar to those in anecdysial exocuticle (compare Fig. 5 with Fig. 4B). Perhaps the proteins missing from extracts of the anecdysial layers have been tanned and thereby rendered insoluble in guanidine thiocyanate as appears to have happened to a 48 kDa protein band. It was prominent in extracts from comparable regions of the exoskeleton, *i.e.*, unpigmented branchiostegites of metecdysial animals (Fig. 3, lane 4), but was absent from branchiostegites of anecdysial animals (Fig. 3, lane 2), suggesting that it had been altered during tanning of the

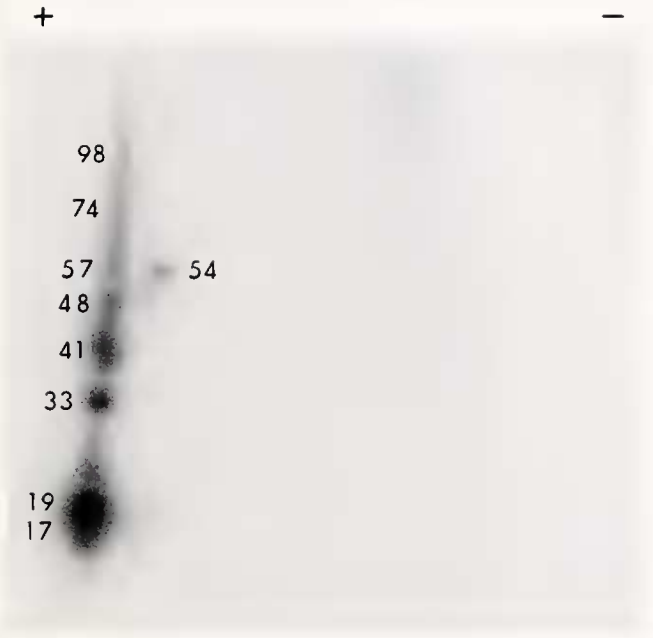

Figure 5. Proteins from newly synthesized metecdysial epicuticle + exocuticle of *Gecarcinus lateralis* have different charges from those in the same layers of anecdysial animals. Autoradiograph of 2D gel; SDS gel for second dimension as in Figure 4; 1×10^6 cpm, 1.1 μg proteins. Autoradiograph exposed 96 h. Approximate protein sizes are indicated (kDa).

exoskeleton and was no longer soluble. Similar changes in solubility have been reported for some cuticular proteins of the meal worm *Tenebrio molitor* (Roberts and Willis, 1980b). These proteins may play an important role during hardening of the exoskeleton. Gunthorpe *et al.* (1990) suggested that newly synthesized proecdysial crab exoskeleton contains unbound proteins that inhibit calcification, and that the cross-linking of these proteins to chitin following ecdysis creates a configuration that promotes calcification.

Prevalence of small proteins

The protein composition of the exocuticle, endocuticle and membranous layer of the anecdysial *G. lateralis* exoskeleton is dominated by small proteins (Figs. 1, 2, and 4; Table II), as are particular layers of some insect cuticles (Fristrom *et al.*, 1978; Roberts and Willis, 1980a; Cox and Willis, 1985; Silvert, 1985; Riddiford *et al.*, 1986; Wolfgang *et al.*, 1986; Horodyski and Riddiford, 1989). Except for the epicuticular quintet, the smallest proteins extracted from exoskeletons of anecdysial land crabs yielded the most intense protein bands on gels stained with either Coomassie blue or silver or were ^{125}I-labeled (Figs. 1, 2, and 4). Although the small proteins are seen as only a few very intense bands on 1D gels (Figs. 1 and 2), on 2D gels they separate into numerous proteins, differing from one another by net charge (charge trains; Fig. 4B–D). Gels of iodinated proteins are shown in Figure 4; similar results were obtained when gels were stained with Coomassie blue (data not shown). In part because the protein bands 28 kDa or smaller in 1D gels are seen to be composed of multiple components in 2D gels, the number of proteins 31 kDa or smaller exceeds those larger than 31 kDa for the three internal layers of the anecdysial exoskeleton (Tables II, III).

Interspecific similarities

Most of the proteins extracted from exocuticle, endocuticle, and membranous layer of the three crabs other than *G. lateralis* were also small. With few exceptions, the most intense bands on silver-stained 1D gels were 31 kDa or smaller (Fig. 6). Similarly sized protein bands (25, 23, 16, 14 and 10 kDa) were seen in extracts from a number of these layers; proteins 31 kDa or smaller showed very similar patterns in the membranous layer of all four species. Again, this similarity is only a suggestive preliminary observation; it does not necessarily imply their identity. Several of these small proteins, which appear as single bands on 1D gels, formed charge trains on 2D gels (data not shown) similar to those formed by some of the smaller proteins of *G. lateralis* (Fig. 4B, C). As was seen with *G. lateralis*, extracts of epicuticles were more likely to have unique proteins (Fig. 6).

Proteins that bind $^{45}Ca^{++}$ in vitro

In addition to M_r and pI, a third property that can be used to distinguish proteins is Ca^{++} binding *in vitro* (not implying Ca^{++} binding *in vivo*). Proteins rich in acidic amino acids may exhibit nonspecific affinity for calcium *in vitro* (Maruyama *et al.*, 1984). For example the 21 kDa size marker, soybean trypsin inhibitor, bound $^{45}Ca^{++}$ (Fig. 7, lane 1) even though its inhibitory activity is not Ca^{++}-dependent (Kunitz, 1947; Laskowski, 1955). We have used the *in vitro* binding of calcium by exoskeletal proteins as a characteristic to distinguish between similarly sized proteins found in extracts of several of the different exoskeletal layers.

The anecdysial epicuticle contained more than three times as many proteins that bound $^{45}Ca^{++}$ *in vitro* as exocuticle or endocuticle. In extracts of anecdysial layers, $^{45}Ca^{++}$ bound to 13 epicuticle protein bands, ranging in size from 141 to 14 kDa, to three exocuticle protein bands, five endocuticle protein bands, and to a 25 kDa protein band from membranous layer. Extracts of newly synthesized metecdysial epicuticle plus exocuticle had more proteins that bound $^{45}Ca^{++}$ in well defined bands than did layers from exoskeletons of anecdysial animals; proteins ranged in size from 71 to 15 kDa (Fig. 7). Six of these metecdysial protein bands (71, 66, 59, 57, 27, and

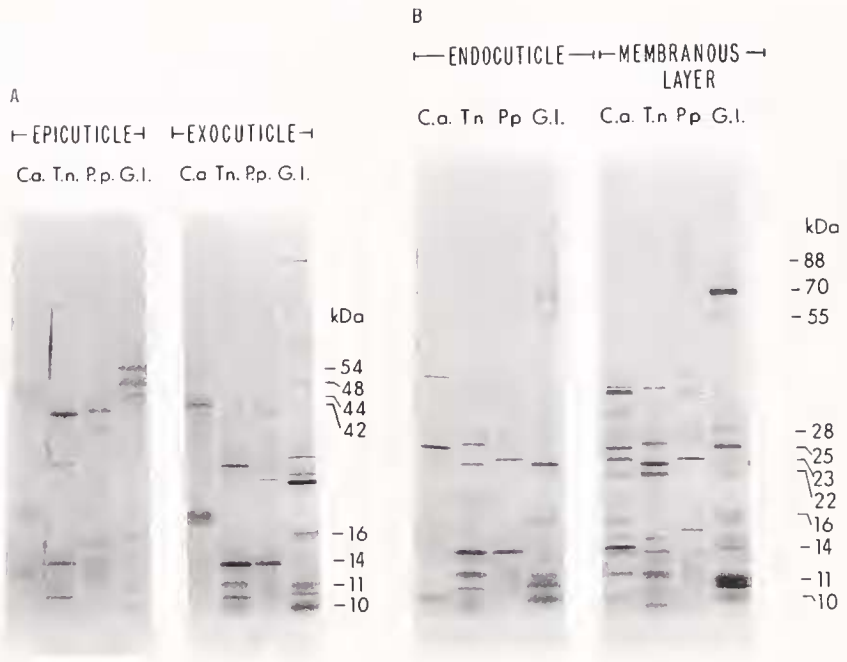

Figure 6. Proteins in the exoskeletal layers of four species of brachyurans. 9–18% 1D SDS-PAGE of proteins extracted from layers of exoskeletons of anecdysial crabs. Gels as in Figure 2; stained with silver. *Cancer antennarius,* C.a.; *Taliepus nuttalli,* T.n.; *Pugettia producta,* P.p.; *Gecarcinus lateralis,* G. l. (A) Epicuticle and exocuticle (B) endocuticle and membranous layer.

24 kDa; Fig. 7, lanes 2 and 3) were absent from extracts of anecdysial epicuticle and exocuticle. Conversely, a 14 kDa protein band in separate extracts of epicuticle, exocuticle, or endocuticle of anecdysial animals bound $^{45}Ca^{++}$, while a similarly sized protein in metecdysial extracts did not (Fig. 7, compare lanes 2–4 with lane 6). Determination of a number of amino acids of the two 14 kDa proteins should indicate whether more than one protein is present (see Willis, 1989).

The presence of many more proteins that bind Ca^{++} *in vitro* in metecdysial extracts than in anecdysial extracts of comparable layers suggests that proteins are altered during the hardening of the exoskeleton, as discussed above. The biological significance of *in vitro* Ca^{++} binding requires further investigation.

Individuality of certain layers of the exoskeleton

Epicuticle has its own specific complement of proteins, while many of the proteins from the exocuticle and endocuticle are similar to each other and to proteins of the membranous layer in both M_r and pI (Table II). Travis (1960) referred to the membranous layer as "the fourth major layer" of the crustacean exoskeleton and noted that it was distinguishable from endocuticle in: (1) staining properties, (2) thickness of its lamellae, and (3) absence of calcium. Babu *et al.* (1985) refer to the membranous layer as the "lower one-fourth region" of the endocuticle. Even though membranous layer appears to share a high fraction of its protein complement with endocuticle and a considerable number of proteins with exocuticle (Table II), it contains proteins not seen in other layers, *i.e.,* a 24 kDa charge train, large amounts of 88 and 28 kDa proteins, of which there are only traces in other layers, and a 25 kDa protein that binds Ca^{++} *in vitro* (Fig. 7). These differences suggest that it is more than simply an uncalcified endocuticle.

Significance of the similarity of proteins in the membranous layer of all four brachyuran species

P. producta, T. nuttalli, and *C. antennarius* are marine crabs, while *G. lateralis* is one of the few brachyurans to have adapted to a terrestrial habitat. Yet proteins of the same sizes (31 kDa or smaller) and similar pI's are found in the membranous layer of all four brachyurans examined despite the evolutionary distance of their taxa (Sections: Oxyrhyncha, Cancridea, and Brachyrhyncha). The protein patterns of all four exoskeletal layers of the two majids (*T. nuttalli* and *P. producta*) are much alike. However, except for the presence of several similarly sized small M_r proteins in layers other than the membranous layer, in *G. lateralis* and *C. antennarius,* exoskeletal protein patterns are recognizably different; they are also different when compared to those of the two majids (Fig. 6). Relationships and homologies of the proteins in the exoskel-

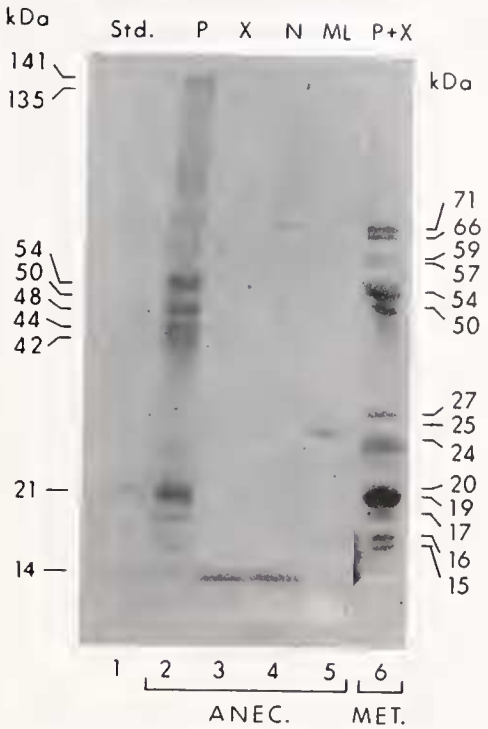

Figure 7. Ca^{++} binding by exoskeletal proteins of *Gecarcinus lateralis*. Autoradiograph of blot of exoskeletal proteins exposed to $^{45}Ca^{++}$. SDS gel as in Figure 2 except 75–100 μg proteins/lane. Abbreviations as in Figure 1. Lane 1, low molecular weight standards (Bio-Rad), Std. Lanes 2–5, proteins from anecdysial animal; lane 2, P; lane 3, X; lane 4, N; lane 5, ML; lane 6, proteins from P + X combined of metecdysial animal. Proteins had been electrotransferred to 0.1 μm (pore size) nitrocellulose. Autoradiograph exposed 15 days. The 21 kDa size marker that binds $^{45}Ca^{++}$ is soybean trypsin inhibitor.

etal layers in different crustacean species await at least partial amino acid analyses as have been performed for a number of insect cuticular proteins (Willis, 1987, 1989).

There is a possible reason for the evolutionary conservation of these small proteins. All arthropods undergo apolysis prior to forming a new exoskeleton and, in brachyurans, the membranous layer is the site at which apolysis occurs. As described above, a number of proteinases have evolved that appear to be active in the degradation of the exoskeleton that results in apolysis (O'Brien *et al.*, 1986; O'Brien and Skinner, 1987, 1988). These proteinases and possibly others degrade almost all of the proteins 31 kDa or smaller. As described above, some large M_r proteins such as the 94–80 kDa group prominent in exocuticle and endocuticle are degraded during proecdysis (Fig. 2). Data presented here show that, rather than being degraded during proecdysis, many of the large M_r proteins are shed with the exuvia (Fig. 2, lanes 6–9) in which they are greatly enriched in comparison with the proteins 31 kDa or smaller. Thus, these organisms appear to have evolved proteinases that degrade the major proteins of the membranous layer, but do not degrade as many or as much of the larger M_r proteins found in other layers.

Acknowledgments

We thank Adrian Wenner for laboratory space at the University of California, Santa Barbara, and Nette Crowe and Jim Hickey for preparing the figures. This research was supported by grants from the National Science Foundation and the Exploratory Studies Program of the Oak Ridge National Laboratory to DMS and by the Office of Health and Environmental Research, U. S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

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